LightMix® Kit *Clostridium difficile* EC

Cat.-No. 40-0573-32 **Change to universal Extraction Control Target (^{n}ECT)**

Kit with reagents for the detection of *Clostridium difficile* and identification of the tcdC deletion variant using the Roche Diagnostics LightCycler® 1.x / 2.0 / 480 II Instruments or cobas z 480 Analyzer.

Lyophilized mix of primers and probes for a total of 96 reactions with a final volume of 20 µl each. **Store protected from light at room temperature (18-25°C), do NOT freeze!**

Instructions for use with the LightCycler® 1.x / 2.0 Instruments see pages 4-5
Instructions for use with the LightCycler® 480 II Instrument / cobas z480 Analyzer see pages 6-7

1. Introduction

Clostridium difficile is a Gram-positive, anaerobic spore-forming bacterium that was identified as an aetiological agent of antibiotic-associated pseudomembranous colitis in the late 1970. It is believed to be responsible for 15-20% of antibiotic-related cases of diarrhea and nearly all cases of pseudo-membranous colitis. Over the last decade, the incidence of *C. difficile*-associated disease has progressively increased and is now a significant clinical problem in North America and Europe.

C. difficile can cause symptoms ranging from diarrhea to life-threatening inflammations of the colon. According to the US Centers for Disease Control and Prevention, each year *C. difficile* is responsible for tens of thousands of diarrhea cases and at least 5,000 deaths.

Preferred targets for the Real-Time-PCR detection are the toxin genes toxA, toxB and toxC[1] and more recently the toxin regulator gene tcdC [2]. An 18 bp sequence deletion in the tcdC gene found in the strain BI/NAP1/027 (ribotype 027) has been associated with an increased morbidity and mortality; however, we do not recommend to make predictions since exceptions have been reported (U. Reischl, pers. comm.).

2. Description

This kit provides a fast and accurate system to detect the *Clostridium difficile* tcdC gene in a nucleic acid extract; the kit includes a spiked Extraction Control (sEC) working also as Internal Control (IC).

A 176 bp long fragment of the tcdC gene - or 158 bp in case of the 18 bp deletion in ribotype 027 - are amplified with specific primers. The resulting PCR fragment is detected with LightCycler® Red 640 labeled hybridization probes (channel 640). The PCR products are identified by running a melting curve analysis. The deletion mutant DNA exhibits a melting point (Tm) of 65.0°C while the wild type shows a broader melting profile with temperatures in the range of 55.0°C to max 65.0°C.

The Control Reaction generates an additional product of 125 bp from the PhHV target, detected with LightCycler® Red 690 labeled hybridization probes (channel 705). This second PCR has no visible impact on the *Clostridium* specific reaction and will even fail in the presence of higher amounts of *Clostridium* (1,000 copies and more).

We recommend to use the 'Extraction Control' procedure; in case that the former procedure shall be maintained the usage as IC is described. Target and control primer/probe sequences remained unchanged. The novel extraction control target ^{n}ECT (no. 30-0259) contains Lamdba and PhHV DNA and may be used also for other LightMix kits, without adding the ECT provided with the other kit(s).
Note: With a MagNA Pure Compact extraction the recovery rate for the EC target can be very low or the DNA target even gets lost.

The use of a color compensation file generated with the LightMix® Kit Color Compensation HybProbe 530/640/690 kit (order no. 40-0318-00) is a prerequisite to run *Clostridium* and Control Reaction.

The supplied standard row of cloned *Clostridium* DNA allows to determine the linear range of the reaction and to estimate the quantity of the target sequence in unknown samples.

Performance testing has been made with the 'LightCycler® FastStart DNA Master HybProbe' only.

3. Set Contents

3	Vials with **green** cap containing lyophilized primers and probes for 32 PCR rxns of *Clostridium*
3	Vials with **white** cap containing premixed lyophilized primers / probes for 32 Control Reactions
1	Vial with **white** cap containing Extraction Control Target (^{n}ECT) 4.8 x 10^6 copies (total)
1	Vial with **black** cap containing the stabilizer for preparation of the No Template Control (NTC)
1	Standard row with 6 lyophilized standards of *C. difficile del.* 10^1 to 10^6 target equivalents per rxn
1	Sealing foil for the standard row
1	Vial with **colorless** cap containing control DNA *C. difficile* 10^5 target equivalents per rxn
1	Vial with **colorless** cap containing control DNA *C. difficile del.* 10^5 target equivalents per rxn
1	Certificate of Analysis (CoA) with lot-specific data

4. Additional Reagents and Items Required

	Roche Diagnostics Cat.-No.
Color Compensation HybProbe order n°40-0318-00	05 997 704 001
LightCycler® FastStart DNA Master HybProbe	03 003 248 001
LightCycler® Capillaries (20 µl) (LightCycler® 1.x / 2.0 Instruments only)	04 929 292 001
LightCycler® 480 Multiwell Plate 384, white (plate based instrument)	Cat.-No. 04 729 749 001
or LightCycler® 480 Multiwell Plate 96, white (plate based instrument)	Cat.-No. 04 729 692 001

For use in LightCycler® 1.x Instruments with software version 3.5.3 read channel F2 instead of channel 640, channel F3 instead of channel 705 for detection. We recommend upgrading to software version 4.1 or higher.

4.1. Optional Additional Reagents

High Pure PCR Template Preparation Kit	Cat.-No. 11 796 828 001
Extraction Control Target (^{n}ECT)	TIB Cat.-No. 30-0259-96

5. Product Characteristics

PCR results (activation, 50 cycles and melting curve) are obtained within 50 minutes with the capillary based LightCycler® 1.x / 2.0 Instruments and within 80 minutes with '480' plate based Instruments.

Sensitivity
These reagents detect 10 copies of *Clostridium difficile* DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' with the LightCycler® 1.x / 2.0 and plate based Instruments (in an exemplary system, using cloned targets as reference).

Measuring range
The linear measuring range of the assay is 10^2 to 10^6 copies of *Clostridium difficile* DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' with the LightCycler® 1.x / 2.0 and plate beased Instruments.

Storage and Stability
- Lyophilized reagents are stable for at least 6 months after shipment when stored protected from light at room temperature (18-25℃). See expiry date o n the product label.
- **Do not freeze** lyophilized reagents.
- Dissolved reagents are stable for at least 10 days when stored protected from light and refrigerated (4℃).
- Dissolved reagents can be long-term stored frozen at -20℃. Avoid multiple thaw-freeze cycles.

6. Experimental Protocol

Start programming before preparing the solutions. See the Instrument operator's manual for details.

6.1. Preparation of Parameter-Specific reagents (PSR) and Control Reaction (EC):

One reagent vial with a **green** cap contains primers and probes to run 32 reactions of *C. difficile.*
One reagent vial with a **white** cap contains primers and probes to run 32 Control Reactions.

Check for the colored pellet, then **add 66 µl** PCR-grade water, mix (vortex) and spin down.

►**Use 2 µl** reagent for a 20 µl PCR reaction.

This solution is stable at least ten days when stored refrigerated at 4°C. Avoid prolonged exposure to light.

6.2. Preparation of Extraction Control Target (ECT):

Add **1,200 µl** PCR-grade water to the vial with **white** cap containing the Extraction Control Target.

6.3. Sample Preparation:

Before extraction add **10 µl** of ECT (vial **white** cap) to the sample. Skip if IC procedure is used.
Perform nucleic acid preparation as described in the protocol of the extraction kit used (**see 4.1**).

6.4. Preparation of No Template Control (NTC) including the Extraction Control Target

Add **900 µl** PCR-grade water to the **NTC** (vial **black** cap) and
add **100 µl** of ECT (vial **white** cap).

►Use **5 µl** No Template Control DNA for a 20 µl PCR reaction.

6.5. Preparation of the Standard Row:

The target DNA is provided in 6 different quantities to yield from 10 to10^6 target molecules in 5 µl once resolved. Start with the lowest concentrated standard (first tube from the extended lip). Use the pipette tip to punch a hole through the sealing foil. Add **40 µl** of **NTC** (vial **black** cap) to each well. Use water if the IC procedure is selected. Mix the target DNA by pipetting the solution up and down 10 times.

►Use 5 µl **standard** for a 20 µl PCR reaction

This standard solution is not long-term stable. Use only fresh prepared solutions only. After adding the target DNA to the reaction mix use the provided sealing foil to close the vials in order to avoid contaminations.

6.6. Preparation of the Positive Control DNA

Add **40 µl NTC** (**black** cap) to the vials with **colorless** cap. Use water for the IC procedure.
Mix by pipetting the solution up and down. ►Use 5 µl control DNA for a 20 µl PCR reaction.

6.7. Preparation of the Reaction Mix

Include Positive Controls and at least one 'No Template Control' (NTC). In a cooled tube, prepare the reaction mix by multiplying the single reaction volumes by the number of reactions plus one reserve :

sEC Procedure	For use with the Roche FastStart HybProbe Master	IC Procedure
Single reaction	**Component**	**Single reaction**
6.6 µl	Water, PCR-grade (colorless cap, provided with the Roche Master kit)	6.35 µl
2.4 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)	2.4 µl
2.0 µl	**PSR** mix (parameter specific reagents, primers and probes, see **6.1.**)	2.0 µl
2.0 µl	**EC** mix (EC reagents containing primers, probes, see **6.1.**)	2.0 µl
---- µl	ECT Control Target (vials **white** cap)	0.25 µl
2.0 µl	Roche Master (red cap, for preparation see Roche manual)	2.0 µl
15.0 µl	**Volume of reaction mix**	**15.0 µl**
15.0 µl	Volume of reaction mix	**15.0 µl**

Table 1

Mix gently, spin down and **transfer 15 µl** of the reaction mix to a capillary or well.
Add 5 µl of sample or standard to each capillary or well for a final reaction volume of 20 µl.
Close the capillaries / attach a foil to the multiwell plate and seal, and spin down.
Start run.

7. LightCycler® 1.x / 2.0 Instruments

7.1. Programming

The protocol consists of four program steps

- 1: Denaturation: sample denaturation and enzyme activation
- 2: Cycling: PCR-amplification of the target DNA
- 3: Melting: melting curve analysis for identification of the PCR product derived from the target DNA
- 4: Cooling: cooling the instrument

Program Step:	Denaturation	Cycling			Melting			Cooling
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	50			1			1
Target [°C]	95	95	62	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:05	00:00:15	00:00:20	00:00:20	00:00:00	00:00:30
Ramp Rate [°C/s]	20	20	20	20	20	20	0.2	20
Sec Target [°C]	-	-	55	-	-	-	-	-
Step Size [°C]	-	-	0.5	-	-	-	-	-
Step Delay (Cycles)	-	-	1	-	-	-	-	-
Acquisition Mode	None	None	Single	None	None	None	Cont	None

Table 2

7.2. Data Analysis

Switch the color compensation mode on. If this mode is not enabled run the color compensation program. Follow the instructions in the manual 'LightMix® Kit – Color Compensation HybProbe'.

Perform data analysis, as described in the LightCycler® Instrument operator's manual.

We recommend using the Second Derivative Maximum method (Automated (F'' max)). The cycle number of the Crossing Point (Cp) of each sample is calculated automatically. The Fit Points method is more-error prone due to the user's influence.

View *Clostridium difficile* data in channel 640, Quantification mode. The negative control (NTC) must show no signal. For the identification of the PCR product view *Clostridium difficile* data in channel 640 Melting Curves mode.

For the Control Reaction, view data in channel 705, Quantification mode. The negative control and the low-concentrated *Clostridium difficile* DNA samples (10 to 1,000 copies) should show an amplification curve for the Control Reaction with a Cp at approximately cycles 29-32.

The provided standard row of cloned *C. difficile del.* target DNA with concentrations of 10^6 to 10^1 copies / reaction should have Cp values between cycles 17 and 37 (see figure 1).

For use in LightCycler® 1.x Instruments select channel F2 instead of channel 640 and channel F3 instead of channel 705 for detection (LightCycler software version 3.5.3).

7.3. Sample Data – Typical Results

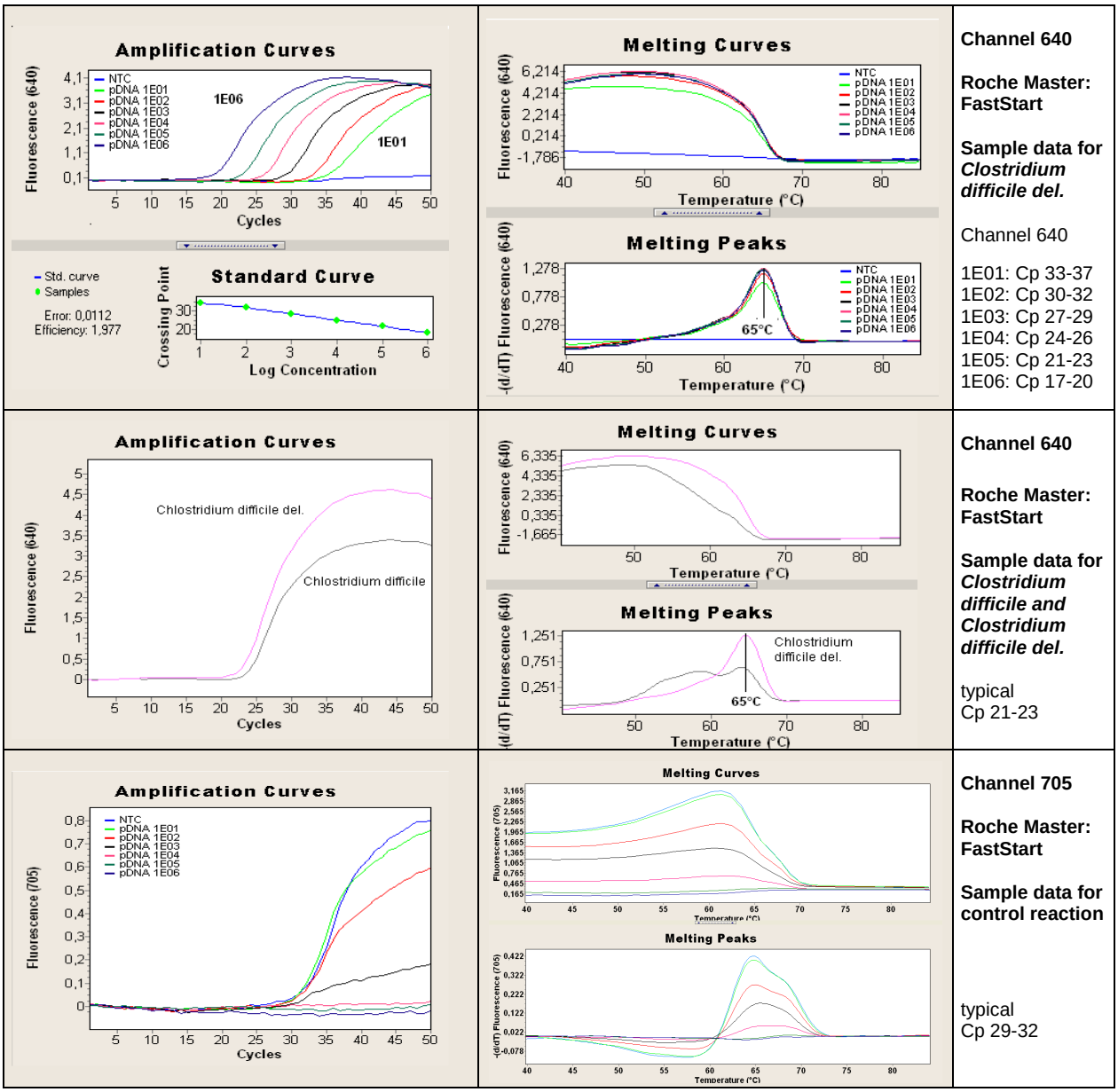

Fig.1. LightCycler® 2.0 sample data for the *Clostridium difficile* detection system.

Upper panels: Left panel channel 640 quantification mode (Second Derivative Maximum) with standard curve for *C. difficile del.* Right panel channel 640 melting analysis for *C. difficile del.*
Central panels: Left panel channel 640 quantification, right panel melting analysis for *C difficile* vs. *Clostridium difficile del.*
Lower panels: Left panel channel 705 quantification for the control reaction, right panel melting analysis (not relevant).

7.4. Interpretation of Data

Sample 640 *Clostridium*	**Sample 705** Control Reaction	**Channel 640 Positive Control**	**Channel 640** Negative Control	**Result** (warnings)
no amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 39[+]	not relevant	amplification	negative	**Positive for *C. difficile***
no amplification	not detectable	amplification	not relevant	PCR failure, repeat experiment
not relevant	not relevant	no amplification	not relevant	PCR failure, repeat experiment
not relevant	not relevant	not relevant	positive	Contamination, repeat experiment

Tab. 3. Typical analysis results (LightCycler® 1.x / 2.0 Instruments, Roche Diagnostics Master: FastStart)

[+] The cut off value is a recommendation only - this value has to be defined by the user. Compare with the lot specific values for 10 copies as reported in the Certificate of Analysis (CoA). 1-2 cycles later than for 10 copies corresponds to ~5 copies/reaction.

8. LightCycler® 480 II Instrument and cobas z 480 Analyzer

8.1. Programming

The protocol consists of four program steps

- 1: Denaturation: sample denaturation and enzyme activation
- 2: Cycling: PCR-amplification of the target DNA
- 3: Melting: melting curve analysis for identification of the PCR product derived from the target DNA
- 4: Cooling: cooling the instrument

Detection Format:
LightCycler® 480 II Instrument: 465-510, 498-640, 498-660
cobas z 480 Analyzer: 465-510, 498-645, 498-700

Program Step:	Denaturation	Cycling			Melting			Cooling
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	50			1			1
Target [°C]	95	95	62	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:05	00:00:15	00:00:30	00:02:00	00:00:00	00:00:30
Ramp Rate [°C/s] **96**	4.4	4.4	2.2	4.4	4.4	1.5	-	1.5
Ramp Rate [°C/s] **384**	4.6	4.6	2.4	4.6	4.6	2.0	-	2.0
Sec Target [°C]	-	-	55	-	-	-	-	-
Step Size [°C]	-	-	0.5	-	-	-	-	-
Step Delay (Cycles)	-	-	1	-	-	-	-	-
Acquisition Mode	None	None	Single	None	None	None	Continuous	None
Acquisitions [per °C]	**-**	**-**	**-**	**-**	**-**	**-**	**1**	**-**

Table 4

8.2. Data Analysis

Note: cobas z 480 Analyzer signal levels are about 50% compared to LightCycler® 480 II results.

Switch the color compensation mode on. If this mode is not enabled run the color compensation program. Follow the instructions in the manual 'LightMix® Kit – Color Compensation HybProbe'.

Perform data analysis, as described in the LightCycler® Instrument operator's manual.

We recommend using the Second Derivative Maximum method (Automated (F'' max)). The cycle number of the Crossing Point (Cp) of each sample is calculated automatically. The Fit Points method is more-error prone due to the user's influence.

View *Clostridium difficile* data with Filter Combination 498-640 (498-645), Quantification mode. The negative control (NTC) must show no signal. For the identification of the PCR product view *Clostridium difficile* data with Filter Combination 498-640 (498-645), Melting Curves mode.

For the Control Reaction view data with Filter Combination 498-660 (498-700), Quantification mode. The negative control and the low-concentrated *Clostridium difficile* DNA samples (10 to 1,000 copies) will show an amplification curve for the Control Reaction with a Cp at approximately cycles 29-32.

The provided standard row of cloned *C. difficile* target DNA with concentration from 10^6 to 10^1 copies / reaction should have Cp values between cycles 17 and 37 (see figure 2).

8.3. Sample Data – Typical Results

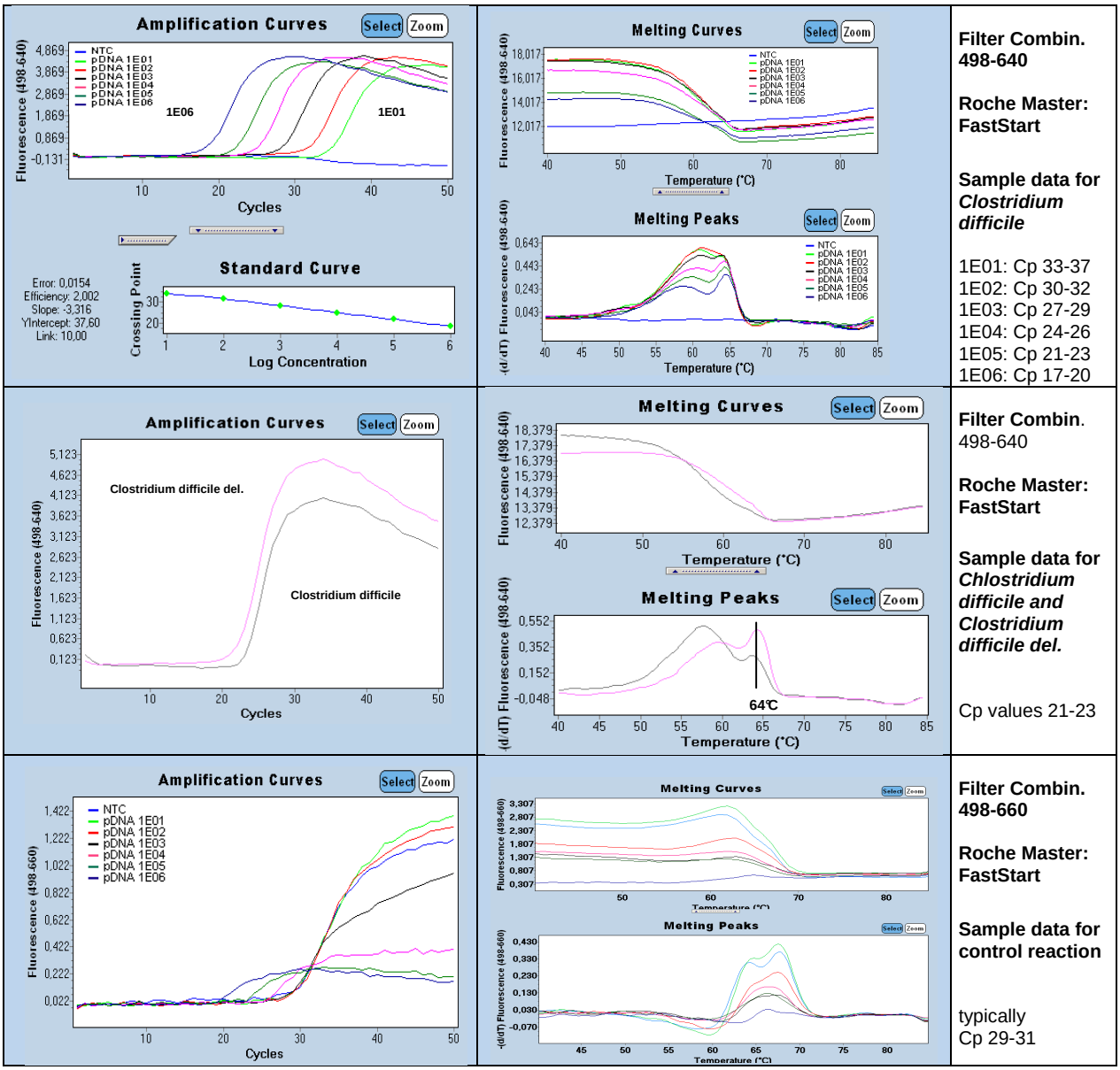

Fig.2. LightCycler® 480 II sample data for the *Clostridium difficile* detection system.

Upper panels: Left panel Filter Combination 498-640 (645) quantification mode (Second Derivative Maximum) with standard curve for *C. difficile del.* Right panel Filter Combination 498-640 (645) melting analysis for *Clostridium difficile del.*
Central panels: Left panel Filter Combination 498-640 (645) quantification mode (Second Derivative Maximum) with amplification curves for *C difficile* and *Clostridium difficile del.* Right panel Filter Combination 498-640 (645) melting analysis for *C. difficile* and *C difficile del.*
Lower panels: Left panel Filter Combination 498-660 (700) quantification mode (Second Derivative Maximum) for the control reaction. Right panel Filter Combination 498-660 (700) melting analysis (not relevant for detection).

8.4. Interpretation of Data

Filter 498-640 *Clostridium*	**Filter 498-660** Control Reaction	**Filter 498- 640 Positive Control**	Filter 498-640 Negative Control	**Result** (warnings)
no amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 39[+]	not relevant	amplification	negative	**Positive for *C. difficile***
no amplification	not detectable	amplification	not relevant	PCR failure, repeat experiment
not relevant	not relevant	no amplification	not relevant	PCR failure, repeat experiment
not relevant	not relevant	not relevant	positive	Contamination, repeat experiment

Tab. 5. Typical analysis results (LightCycler® 480 II Instrument, Roche Diagnostics Master: FastStart)
[+] The cut off value is a recommendation only - this value has to be defined by the user. Compare with the lot specific values for 10 copies as reported in the Certificate of Analysis (CoA). 1-2 cycles later than for 10 copies corresponds to ~5 copies/ reaction.

9. Evaluation Study Results - Specificity (Inclusivity and Exclusivity)

Horridge et al.[3] compared 5 commercial kits and reported a diagnostic sensitivity of 93.3% (other kits max. 86.7%), a specificity of 99.7%, a positive predictive value (PPV) of 93.3% and a NPV of 99.7%.

10. Conversion Factor

PCR reports copies / reaction. Conversion to copies / mL depends on sample and extraction volumes:

VL [copies/ml] = MV x EVF x SF

where:

VL	**=**	**Copies per mL sample**
MV	=	**M**easured **V**alue [copy number per reaction]
EVF	=	**E**xtraction **V**olume **F**actor [Final extraction volume / PCR sample volume]
SF	=	**S**ample **F**actor [1,000 µl / extracted volume of clinical sample]

As an example, extracting 200µl sample results in a correction factor of 5. Using 5 µl from a total eluation volume of 100 µl results in a correction factor of x 20, resulting in a conversion factor of 100.

11. References

[1] Rapid Detection of *C. difficile* in Feces by Real-Time PCR, Belanger et al. J Clin Microbiol 41, 2003, 730-734

[2] Comparison of a Real-time PCR for Detection of the tcdC Gene With Four Toxin Immunoassays and Culture in the Diagnosis of *Clostridium difficile* Infection. Sloan et al. J Clin Microbiol. 2008

[3] An evaluation of laboratory methods for the diagnosis of toxigenic *C. difficile* infection: enzyme immunoassays for glutamate dehydrogenase and toxins A and B, real time PCR (tcdC) and stool culture, Horridge et al. NZ J Med Lab Sci 2011

[4] Use of the LightMix kit *C. diff* as reference assay: A Cost-Effective Approach for Detection of Toxigenic *Clostridium difficile*: Toxigenic Culture Using ChromID *Clostridium difficile* Agar. Luk et al., JCM (2014)

12. Material Safety Data

According to OSHA 29CFR1910.1200, Australia [NOHSC:1005, 1008 (1999)] and the European Union Directives 67/548/EC and 1999/45/EC any products which not contain more than 1% of a component classified as hazardous or classified as carcinogenic do not require a MSDS.

Product is not hazardous, not toxic, not IATA-restricted. Product is not from human, animal or plant origin. Product contains synthetic oligonucleotide primers and probes.

10. Version History

Red notes mark changes in procedures, blue modification of sequences

V100825	Instructions for Lightcycler® 1.x / 2.0 / 480 II
V111107	Adaption of Cp values and implementation of version history
V130813	z 480 included, MSDS included, Roche color compensation discontinued
V140714	Internal control changed to extraction control, Kit change to 3 x 32 rxn Control target changed from Lambda DNA to PHhV (cloned)
V140909	Editorial changes
V150505	Clinical evaluation data added (ref. 3)
V151015	Change to universal nECT target containing Lambda and PhHV DNA
V160223	Control Reaction Target information is PhHV

Roche SAP order n° 05945313001

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Notice to Purchaser

These reagents were developed and manufactured by TIB MOLBIOL GmbH, Berlin, Germany..